Characterization of the role of pneumococcal beta-galactosidase, BgaA, in adherence

A Senior Honors Thesis

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ABSTRACT

Streptococcus pneumoniae is a major human pathogen that causes over 1.6 million deaths worldwide each year through such diseases as pneumonia, meningitis, and otitis media (1). Adherence of *S. pneumoniae* to the airway is essential for colonization which is a precursor to disease; however, adherence mechanisms are poorly understood. We discovered a novel mechanism that involves the adherence of beta-galactosidase, BgaA (a sugar-cleaving enzyme) to the epithelial cell surface. Preliminary studies suggest that the epithelial cell receptor for BgaA is a glycosphingolipid. Asialo-GM1, a glycosphingolipid previously proposed as a receptor for *S. pneumoniae*, was tested as a possible receptor for BgaA; however, the addition of neither purified asialo-GM1 nor anti-asialo-GM1 was able to reduce adherence of *S. pneumoniae* to epithelial cells, indicating that asialo-GM1 is likely not a receptor for BgaA. When demonstrating clinical relevance of this novel mechanism, some clinical isolates were found to be reduced in adherence after the addition of recombinant BgaA (rBgaA), whereas other clinical isolates were not. Differences in adherence could be attributed to allelic diversity within the gene or differences in enzyme expression, among other possibilities. To determine if allelic diversity may contribute to BgaA-mediated adherence, the gene was sequenced. Predicted amino acid differences were found between two strains reduced in adherence upon the addition of rBgaA and two strains that were not, indicating that these differences could contribute to the ability of BgaA to act as an adhesin. The BgaA alleles were exchanged between strains; however, these mutants did not show an exchange in phenotype, indicating that these sequence differences are not responsible for the ability of BgaA to act as an adhesin. To determine if increased levels of BgaA expression contribute to adherence, beta-galactosidase activity was tested and used as an indicator of possible expression level differences. Strains reduced in

adherence upon the addition of rBgaA had higher activity, suggesting increased expression of BgaA. These findings will help to further characterize the BgaA-mediated mechanism of adherence which will help in understanding pneumococcal colonization.

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CONTENTS

INTRODUCTION

Streptococcus pneumoniae is a major human pathogen that causes diseases ranging from otitis media to bacteremia and meningitis and is the most common bacterial cause of community acquired pneumoniae (10). *S. pneumoniae* is estimated to cause over 1.6 million deaths worldwide each year with more than 45,000 deaths occurring annually in the U.S. (24). Acting as an opportunistic pathogen, *S. pneumoniae* is more likely to cause disease in young, elderly, or immuno-compromised individuals (7). Despite the high levels of morbidity and mortality, *S. pneumoniae* generally colonizes the oropharynx or nasopharynx asymptomatically (33) and is usually cleared by the host; however, invasive disease can occur if bacteria spread beyond the upper airway to areas such as the lungs, blood, or meninges.

Over 90 serotypes, as defined by capsule polysaccharide, of *S. pneumoniae* are known; however, most disease is caused by a small number of serotypes (27). Two vaccines based on capsule polysaccharide are currently available. Created in 1983, the first vaccine was composed of purified free polysaccharides derived from the capsule of 23 serotypes (8). This vaccine, however, lacks the ability to induce immunological memory and is therefore limited in its efficacy (3). In 2000, a conjugated vaccine linking capsular polysaccharide of seven pneumococcal serotypes to a protein carrier was introduced (2). Able to elicit a memory response in young children (5), this vaccine has helped to decrease invasive disease and colonization due to capsular types included in the vaccine (17, 21, 26). Despite the ability of the PCV7 to reduce disease caused by vaccine strains, colonization and disease caused by non-vaccine strains has significantly increased in North American populations (17, 21) (11, 25). This evidence indicates that efficacy of the PCV7 vaccine will likely decrease over time. Another

conjugate vaccine containing up to 13 serotypes is in development to improve pneumococcal protection in adults and children (2); however, efficacy of this vaccine is likely to decrease over time, as well. Similarly, pneumococcal strains have become increasingly resistant to antibiotics (9, 16). Limited vaccine efficiency and increasing antimicrobial resistance indicate that research contributing to possible treatments and/or preventative measures is of importance.

As previously stated, pneumococcal colonization is essential to cause disease. Despite years of research, little is known about how *S. pneumoniae* establishes and maintains colonization. Adherence of the bacteria to the epithelial cell surface is known to play a role in colonization and several mechanisms have been already been identified (13). Because adherence is an important step in colonization, *S. pneumoniae* is likely to use multiple mechanisms. Our lab has identified a novel mechanism of adherence involving pneumococcal surface-associated exoglycosidases (terminal sugar-cleaving enzymes). Pneumococcal exoglycosidases are presumed to cleave sugar structures that decorate the epithelial cell surface in the human airway. The released sugars are proposed to be used as a carbon source for pneumococcal metabolism (6). Cleavage of these sugars has also been proposed to help the pathogen move through the thick mucin layer (28, 36) or help to reveal receptors for adherence (30, 31). NanA, a pneumococcal cell surface-associated exoglycosidase that cleaves terminal sialic acid, has been proposed to reveal a receptor on the epithelial cell surface. Tong et al. 2002 (31) demonstrated increased pneumococcal adherence to neuraminidase-treated chinchilla tracheas. Similarly, Barthelson et al.1998 (4) demonstrated that adherence to epithelial cells was inhibited by the addition of sialyated glycoconjugates. *S. pneumoniae* encodes other surface-associated glycosidases that could further modify host glycoconjugates. We recently demonstrated that *S. pneumoniae* can

sequentially deglycosylate N-linked glycans through the activity of NanA, β-galactosidase, BgaA, and N-acetylglucosaminidase, StrH (19). The ability of *S. pneumoniae* to sequentially deglycosylate host glycoconjugates suggests that other exoglycosidases could be working in conjunction with NanA to reveal a receptor for pneumococcal adherence on the epithelial cell surface. While investigating the role of sequential deglycosylation in revealing receptors, NanA and BgaA, but not StrH, were determined to be necessary for pneumococcal adherence (19). This evidence suggested that NanA and BgaA act sequentially to reveal a receptor. Adherence of a *nanA* mutant to epithelial cells was complemented by the addition of exogenous neuraminidase; however, adherence of a *bgaA* mutant to epithelial cells could not complemented by the addition of recombinant BgaA (rBgaA) (Unpublished data). This evidence suggests that NanA cleaves terminal sialic acid, revealing a receptor to which BgaA binds. Evidence supporting this hypothesis includes: The addition of rBgaA reduced the binding of wildtype strains to human epithelial cells and the adherence of rBgaA coated fluorosphere beads to epithelial cells indicated that BgaA can bind directly to epithelial cells. Lastly, a BgaA active site mutant was constructed and tested in adherence and was not significantly reduced in adherence as compared to wild-type, further demonstrating that the role of BgaA in adherence is independent of enzymatic activity (Unpublished data).

BgaA is the second-largest protein encoded in the pneumococcal genome and is approximately twice the size of other β-galactosidases at 2228 amino acids, suggesting that BgaA may have a second function (23). BgaA is predicted to be secreted and attached to the pneumococcal surface in a sortase-dependent manner (18) and other than the cell-wall anchoring motif, the function of the C-terminal region remains unknown. *Bifidobacterium bifidum* and *S. gordonii* express β-

galactosidases of similar length to *S. pneumoniae* (12, 32) and although the role of these proteins in adherence has not been tested, it is possible that BgaA belongs to a family of adhesins.

The host cell receptor for BgaA remains unknown. The contribution of NanA to this mechanism suggests that the receptor is a glycoconjugate (a sugar structure attached to another molecule), such as a glycoprotein or glycosphingolipid. To determine of BgaA binds to a glycoprotein, adherence of *S. pneumoniae* to protease-treated human epithelial cells (to cleave possible glycoprotein receptors) was measured. Bacterial adherence was significantly higher to protease treated epithelial cells as compared to non-protease treated epithelial cells, indicating that the receptor is not a glycoprotein cleaved by protease. In fact, the increase in bacterial adherence to protease-treated epithelial cells suggests that protease treatment helped to further reveal an epithelial cell receptor for *S. pneumoniae*. This data indicate that the receptor could instead be a glycosphingolipid. In investigation of bacterial adherence to the carbohydrate sequence GalNAcβ1-4Gal found in some glycolipids, Krivan et al. demonstrated the ability of S. pneumoniae to bind to the glycosphingolipid, asialo-GM1 (20). The galactose residue in asialo-GM1 may help mediate the binding of BgaA, which has enzymatic specificity for terminal galactose.

In demonstrating clinical relevance, the adherence of some recent clinical isolates obtained from Nationwide Children's Hospital to epithelial cells were found to be blocked by the addition of rBgaA whereas some other clinical isolates were not (Figure 1). The reasons for differences in the contribution of BgaA to adherence could be accounted for by many reasons including possible amino acid sequence differences within the BgaA allele amongst the strains, possible

BgaA expression level differences, possible differences in capsular expression, or the distribution of other adherence mechanisms amongst the strains. Amino acid sequence differences may contribute to differences in the contribution of BgaA to adherence if these differences affect binding of the receptor. Or, differences in contribution of BgaA to adherence could result from differences in the level of protein expression on the bacterial cell surface, as a higher expression of BgaA could mean that more protein is available to act as an adhesin. Similarly, differences in capsular expression could account for differences in the contribution of BgaA to adherence as a thinner capsule could mean that more protein is available to act as an adhesin. Lastly, because adherence is a preliminary step to colonization and pneumococci are diverse, different strains could encode different redundant mechanisms of adherence.

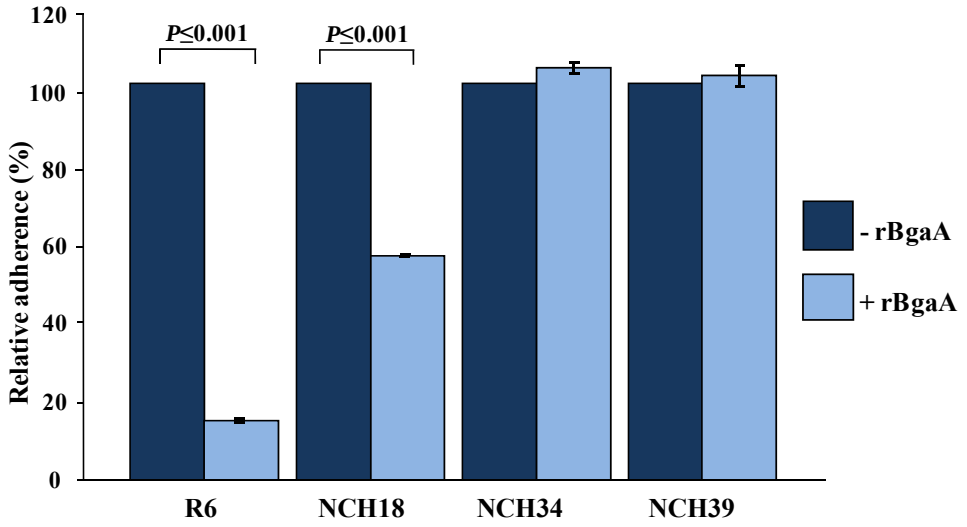

Figure 1. Demonstration that the BgaA-mediated mechanism is relevant *in vivo*, however differences in the ability of BgaA to act as an adhesion differ among recent clinical isolates. Adherence of strains (O.D. 600 nm = 0.6) to D562 cells over 60 min is expressed as a percentage of parental strain adherence. All wells were pretreated for 30 min with 0.01 U of purified *C. perfringens* neuraminidase per 1.9 cm^2 of well surface area. Where indicated, 0.433 pm of rBgaA was added to the inoculum. Values are the mean of at least three independent experiments ±SD. Statistical significance was determined using Student's *t*-test.

Despite the importance of adherence to pneumococcal colonization and disease, most mechanisms remain unknown. We have discovered a novel mechanism of adherence involving the pneumococcal surface-associated exoglycosidases, NanA and BgaA. The aim of this study was to further characterize the BgaA-mediated mechanism of adherence so as to better understand pneumococcal adherence and help in the ability to develop more effective strategies to reduce pneumococcal colonization and disease.

METHODS

Bacterial strains, culture, media, and chemicals. Wild-type and genetically altered strains of *S. pneumoniae* and the recent clinical isolates used in this study are described in Table 1. Broth cultures were grown at 37°C in Todd-Hewitt broth (Becton, Dickenson, and Co., Sparks, MD) supplemented with 0.2% w/v yeast extract (Becton, Dickenson, and Co.) (THY). C media with 5% yeast extract (C+Y) pH 8 was used for transformations (22). *S. pneumoniae* was also grown at 37°C and 5 % CO_2 overnight on tryptic soy (TS) (Becton, Dickenson, and Co.) plates with 1.5 % agar that were spread with 5000 U catalase (Worthington Biochemical Corporation, Lakewood, NJ) prior to plating bacteria. Where appropriate, strains were selected for on TS plates that contained either streptomycin (200 μg/mL) or kanamycin (500 μg/mL) as appropriate. Detroit 562 cells (ATTC CCL-185), a human pharyngeal carcinoma cell line, were grown in minimal essential media supplemented with 10 % fetal bovine serum, 1.1 % sodium pyruvate, 110,000 u/ml penicillin, and 110 mg/ml streptomycin at 5 % CO_2. Unless otherwise indicated, all chemicals, substrates, and enzymes were purchased from Sigma Chemicals (St. Louis, MO).

Genomic DNA Preparation. All genomic DNA was prepared as previously described (35). Briefly, 10 mL of Optical Density at 600 nm (OD_{600}) = 0.8 culture was centrifuged and re-suspended in 500 μL 50 mM ethylenediaminetetraacetic acid (EDTA)/10 mL tris(hydroxymethyl)aminomethane (TRIS) (pH 8.0). Bacteria were incubated at 37°C with 20 μL 10 mg/mL lysozyme for 30 minutes (min) and subsequently with 30 μL 10 mg/mL proteinase K for an additional 30 min. 40 μL of 20 % v/v N-lauroyl sarcosine was added and mixed by inversion. After 10 min bacteria were subjected to two rounds of extraction with an equal volume of phenol/chloroform/isoamyl alcohol (Fisher Scientific, Pittsburgh, PA) and a final

extraction with an equal volume of chloroform. DNA was isolated from the aqueous phase and precipitated with 100 % cold ethanol. When necessary, the addition of 3 mM sodium acetate (pH 5.2) at 1/10 the sample volume was used to aid precipitation. The pellet was washed with 70 % v/v ethanol, vacuum dried, and re-suspended in 50 µL dH_20.

Polymerase Chain Reaction. All reactions (50 µL unless otherwise noted) were carried out in 0.2 mL flat cap PCR tubes. Master-mixes containing all reagents except the DNA template were pre-mixed and aliquoted into PCR tubes; DNA template was added individually to each reaction. H_20 rather than DNA template served as a negative control. When possible, positive controls were included.

Reactions using the high-fidelity polymerase, Pfx50 (Invitrogen, Carlsbad, CA). A high fidelity polymerase was used in the construction of the desired construct for transformation into *S. pneumoniae*. To identify optimal amounts of DNA, neat, 1:5 and 1:10 dilutions of DNA were included until one was identified as most suitable. Primers were generally used at 0.4 µg/µL per manufacturer's instructions; however, if no product was obtained, a primer concentration of 1 µg/µL was used. Standard 50 µL reaction:

Reagent	*Amount (µL)*
10x *Pfx*50 PCR Buffer	5
10x dNTP (2 mM each)	0.75
$MgSO_4$	0.5
Primer 1 (0.4 or 1 µg/µL)	0.2
Primer 2 (0.4 or 1 µg/µL)	0.2
*Pfx*50 polymerase	1
Chromosomal DNA template	0.3
De-ionized H_20	To 50

Thermocycling Parameters in the BioRad MyCycler™ Thermal Cycler

95°C	Denature	4 min	1x
95°C	Denature	1 min	30x
≥60°C	Annealing	1-2 min	
72°C	Elongation	1 min/kb	
72°C	Polishing	1 min	1x
4°C			HOLD

The annealing temperature should be determined for each reaction by estimating the temperature at which 50 % of the primer is dissociated (T_m) and using an annealing temperature 2°C less than that of the lowest primer. Estimation of primer T_m can be performed by counting each cytosine or guanine as 4°C and each adenine or thymine as 2°C.

Reactions using Choice™ Taq DNA Polymerase (Denville, Metuchen, NJ). When amplifying products for sequencing or screening potential mutants with flanking primers, standard *Taq* polymerase was used. Standard 50 µL reaction:

Reagent	*Amount (µL)*
10x PCR Buffer containing 15 mM $MgCl_2$	5
10x dNTP (10 mM each)	0.75
$MgCl_2$	0.5
Primer 1 (1 µg/µL)	0.2
Primer 2 (1 µg/µL)	0.2
Taq polymerase	0.2
DNA template	0.3
De-ionized H_20	To 50

Thermocycling Parameters in the BioRad MyCycler™ Thermal Cycler

95°C	Denature	4min	1x
95°C	Denature	1min	30x
55 ± 5°C	Annealing	1-2min	
72°C	Elongation	1min/kb	
72°C	Polishing	1min	1x
4°C			HOLD

Sequencing Reactions with Big Dye® Terminator reaction mixture. Sequencing was conducted using the PCR products obtained for segments of the *bgaA* region of clinical isolates and from reactions run with flanking primers for exchanged *bgaA* regions. Standard 10 µL sequencing reaction with Big Dye Terminator version 3.1:

Reagent	*Amount (µL)*
DMSO	0.5
DNA	45 ng*
Primer	1.6 pmol (1 µL of 1:100 dilution)
Big Dye	2
5x Buffer	1
De-ionized H_20	To 10

*****Amount of a DNA sample can be estimated by comparing the relative intensity of the template band to a band with a known amount of DNA in the 1 kb ladder when run on an agarose gel.

Sequencing Thermocycling Parameters in the BioRad MyCycler™

96°C	Denature	10 sec	25x
50°C	Annealing	5 sec	
60°C	Elongation	4 min	
4°C			HOLD

Electrophoresis of DNA on a 1 % agarose gel. Agarose gels were prepared by dissolving agarose in 1xTAE to 1 % w/v. Ethidium bromide (final concentration = 0.025 µg/mL) was added directly to the melted agarose. To visualize chromosomal DNA, 2 µL of DNA was mixed with 3 µL dH_2O and 2 µL loading dye. To visualize PCR products (unpurified or purified with the QiaQuick PCR purification kit (Qiagen)) mix 2 µL PCR product with 3 µL dH_2O and 2 µL of loading dye. Samples were electrophoresed horizontally at 110 Volts in gels submerged in 1xTAE. DNA was visualized by UV-transillumination using Kodak Molecular Imaging Software and photographed with a Kodak IS2000MM camera.

Sequence analysis. Upstream and downstream sequences were compared using Lasergene SeqMan software. If a discrepancy was found between sequencing reactions, the chromatographic output was analyzed to determine the correct base. Sequence comparisons were performed using Lasergene MegAlign software.

Transformations. Strains were grown in C+Y medium pH 8 at 37°C to an OD_{600} = 0.15. 50 µL of bacterial culture was transferred to 950 µL aliquots of C+Y pH 8 media warmed to 30°C. Bacterial competence was induced by the addition of stimulating peptide, 2 µL 1 mg/mL CSP1 and 10 µL 10 mM $CaCl_2$ and incubated for 10 min at 30°C. After the addition of DNA, cultures were incubated for 40 min at 30°C and then 90 min at 37°C.

Genetic exchange of *bga*A region. This was performed using a Janus Cassette selection system (29). Construction of a mutant strain using this system requires two rounds of transformation. The first round introduces a Janus *rpsL* cassette, which inserts kanamycin resistance, into the genomes of a streptomycin resistant (Sm^R) R6 and NCH39 *S. pneumoniae* strains, replacing the area of interest. The Janus construct used to transform our *S. pneumoniae* strains was engineered using a variation on the splicing by overlap extension (SOE) by PCR process (14). In order to minimize PCR generated errors, all PCRs were conducted using a high-fidelity proofreading polymerase (*Pfx50*). All PCR products were purified and concentrated using a PCR purification kit. DNA fragments flanking the *bgaA* region were amplified using primers E.1 and E.2 (upstream fragment) and E.3 and E.4 (downstream fragment) (Table 2). Each primer pair contained one hybrid primer such that sequences complementary to the Janus cassette primers

were introduced at the 3' end of the upstream DNA fragment and 5' end of the downstream DNA fragment. These PCR products were sequentially joined to the Janus cassette PCR product (primers J.1 and J.2) using SOE. Transformants were selected for on kanamycin, and their antibiotic susceptibility phenotype was subsequently confirmed as kanamycin resistant (Km^R) and Sm^S Using chromosomal DNA, the second round of transformation replaced the Janus cassette in R6 with DNA from the *bgaA* region of NCH39 and the Janus cassette in NCH39 with DNA from the *bgaA* region of R6. Unmarked mutants were selected for on streptomycin, and their antibiotic susceptibility phenotype was subsequently confirmed as Km^S and Sm^R. Generation of the unmarked mutants was confirmed by PCR with primers flanking the construct (E.5 and E.6) and sequencing.

Adherence of *S. pneumoniae* to human epithelial cells. Strains were grown in THY medium at 37°C to an OD_{600} = 0.6 and diluted 25 fold in minimal essential media (Invitrogen) supplemented with 10 % fetal bovin serum (Invitrogen) and 1.1 % sodium pyruvate (Mediatech, Inc., Manassas, VA). 80 µL of the bacterial innoculum was added per 1.9 cm^2 of well surface. Epithelial cells were pretreated for 30 min with 0.01 U of purified *Clostridium perfringens* neuraminidase per 1.9 cm^2 of well surface area. (*C. perfringens* neuraminidase has the same cleavage specificity as pneumococcal neuraminidase, NanA.) Neuraminidase was removed by three washes in phosphate buffer solution (PBS). The bacteria were allowed to adhere at 37°C and 5 % CO_2 for 60 min. Non-adherent bacteria were removed by five washes with PBS. Epithelial cells and adherent bacteria were lifted with 200 µL 0.25 % trypsin/1mM EDTA (Invitrogen), vortexed and maintained at 4°C. Bacteria were then enumerated by serial dilutions and plating of either 10 µl or 100 µl aliquots in triplicate on TS plates. The percentage

adherence of all strains was calculated as the proportion of the inoculums that adhered to epithelial cells. Adherence of the untreated wild-type and parental strains was adjusted to 100 % and the adherence of other conditions or strains was made relative to this value. Averages of at least three independent experiments performed in triplicate were used for statistical analysis. Assays in which the adherence of clinical isolates was reduced by the addition of recombinant BgaA (rBgaA), 4.33 pm of BgaA per 1.9 cm^2 of well surface area was added to bacterial inoculums. Assays in which adherence of R6 was blocked with anti-asialo-GM1, D562 cells were incubated with 100 µL of 0, 3.7, or 10 mg/ml anti-asialo-GM1 polyclonal rabbit antibody for 60 min after pretreatment with purified neuraminidase and prior to inoculation. Assays in which adherence of R6 was blocked with purified GM1, 10 ng, 100 ng, 10 µg, or 100 µg of purified GM1 was added per 80 µL bacterial inoculum and incubated on ice for 30 min prior to being added to the epithelial cells.

Beta-galactosidase activity assays. Strains were grown in THY medium at 37°C to OD_{600} = 0.6. 1 mL each of total culture was placed in a microcentrifuge tube and 3 µL toluene was added. After being vortexed for 30-40 sec, the tubes were rotated at 37°C and 5 % CO_2 for 15 min. The tubes were then left uncapped in the fume hood for 20 min to ensure evaporation of the toluene. 200 µL of 4 mg/mL o-nitrophenyl-β-D-galactopyranoside (ONPG- the colorimetric substrate) dissolved in 0.1 M sodium phosphate buffer solution was added to each tube which were then placed back in the rotator at 37°C and 5 % CO_2 for 60 min. The reaction was stopped by the addition of 500 µL 1 M sodium carbonate. The tubes were centrifuged and the absorbance of their supernatant measured at 420 nm by the BioTek Synergy HT plate reader.

The activity of BgaA, as expressed in Miller units, can be found by using the following equation: $(100 \times OD_{420})/(t \times V \times OD_{600})$ t= 60 min V=1 mL.

Statistical analysis. Adherence assays and β-galactosidase activity assays were run in triplicate on three independent occasions. Media blanks were subtracted from activity assay data. All presented data was analyzed using Student's *t*-tests.

Table 1. Strains used in this study.

Strain Name	Serotype	Characteristics/genotype	Source or Reference
R6	UN	Lab strain	Ianneli *et al*. (1999) (15)
R6Δ*bgaA*	UN	Δ*bgaA*	Unpublished data
NCH18	22F	Clinical Isolate	Nationwide Children's Hospital
NCH34	35F	Clinical Isolate	Nationwide Children's Hospital
NCH39	35F	Clinical Isolate	Nationwide Children's Hospital
R6Sm^R	UN	Lys56→Thr in *RpsL* conferring Sm^R	This study
NCH39Sm^R	35F	Lys56→Thr in *RpsL* conferring Sm^R	This study
R6*bgaA*NCH39	UN	Contains *bgaA* of NCH39	This study
NCH39*bgaA*R6	35F	Contains *bgaA* of R6	This study

a. UN indicates unencapsulated
b. Sm^R indicates streptomycin resistant

Table 2. Primers used in this study.

Group	Number	5'→3' Sequence
bgaA: sequencing	S.1	AGTACGCAGCAAAAGCAAAATAAT
	S.2	TAGATGCGATTTTAGAAGTTG
	S.3	CAGAGTGGGCAGTAAGGGTGAAT
	S.4	CCAGCGGCAGTTCGTCTTA
	S.5	ATTGGCTGGTTGGTTATTGTAGGA
	S.6	TTTACGCTCCATACGCCATCTCAT
	S.7	TCAAACTACCAAGCCTACGA
	S.8	GAATGTCAGGCGCTTTTATC
	S.9	GCGCAATCCATCCGTCCTGT
	S.10	CAAATCCAAGCGACCCAG
	S.11	GCGGCAGCCAAACAAGGAC
	S.12	GTTTGTCCTTGTTTGGCTGCCGC
	S.13	CTAATCAATTCGTACAAGGCAGG
	S.14	GGCTGAATAAAGATGCTCGCACGC
	S.15	CTGAGAAGACAGTTGCTGCTG
	S.16	CAGTTGACTAAGGAAAAACCAG
	S.17	CCTGCTGCTACTGCTGCTTG
	S.18	TGGCGTCTACATGGATTCTCAAGT
	S.19	TCACCAATATAGTCCGTACCT
	S.20	CACCTGAAATGCCTACCACTGTT
	S.21	GGATGTTGATAAGACTCGCTATG
	S.22	GTAACTACTAATCCTGCACT
Janus	J.1	CGTTTGATTTTTAATGGATAATG
	J.2	GGGCCCCTTTCCTTATGCTT
bgaA: exchange	E.1	ATTCGTAAGTTTGCTGTGGGAG
	E.2	CATTATCCATTAAAAATCAAACGTTTAGCATCTTCCTTGAGCGG[1]
	E.3	AAGCATAAGGAAAGGGGCCCTACAAGAAGATCTTCCAAAACTC[2]
	E.4	GGTCTTTAATGATAAAGAAGGTAT
	E.5	AAGGCCATTGGAATCGG
	E.6	TATTTAGGACAAGAGTTTTTC

a. Accession number AE007374 for all except Primer J.1- AY334019 and Primer J.2- AE005672
b. Underline indicates reverse complement of Primer J.1[(1)] and J.2[(2)]

RESULTS

Data suggest that BgaA does not bind to asialo-GM1.

Previous investigation into possible epithelial cell receptors indicates that the receptor is likely a glycoconjugate, and more specifically a glycosphingolipid. The glycosphingolipid, asialo-GM1 has previously been proposed as a receptor for *S. pneumoniae* (20). To determine if asialo-GM1 is the epithelial cell receptor for BgaA, two methods were used. In the first set of experiments, 10 ng, 100 ng, 10 µL and 100 µL of purified asialo-GM1 was incubated with bacterial inoculums before they were tested in adherence to neuraminidase-treated epithelial cells. If asialo-GM1 binds BgaA, the purified asialo-GM1 should decrease the availability of BgaA on the bacterial cell surface and would presumably reduce the adherence of *S. pneumoniae* to epithelial cells. Similarly, in the second set of experiments, neuraminidase-treated epithelial cells were pretreated with 3.7 and 10 mg/mL of the rabbit polyclonal antibody, anti-asialo-GM1, which binds to and blocks asialo-GM1. If asialo-GM1 is the receptor for BgaA, a reduction in adherence of the bacteria to the pretreated epithelial cells should occur. Neither the addition of purified GM1 (Figure 2) or the pretreatment of epithelial cells with anti-asialo-GM1 (Figure 3) significantly reduced the adherence of R6 to epithelial cells, indicating that asialo-GM1 is likely not a receptor for BgaA. Knowledge of the binding site on the *bgaA* allele would be helpful in determining other possible receptors for BgaA. Amino acid sequence differences could contribute to binding site variability which may account for the differences in the ability of BgaA to act as an adhesin for some recent clinical isolates. Therefore, investigation into allelic variation among the *bgaA* alleles of clinical isolates could prove beneficial.

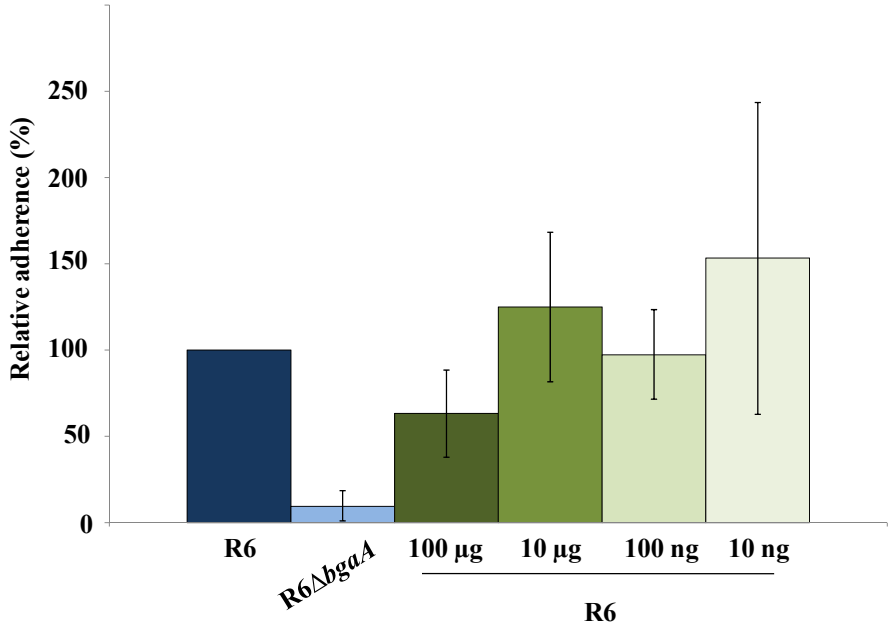

Figure 2. The addition of purified asialo-GM1 does not significantly reduce adherence of R6 to epithelial cells. Adherence of strains (O.D. 600 nm = 0.6) to D562 cells over 60 min is expressed as a percentage of parental strain adherence. All wells were pretreated for 30 min with 0.01 U of purified *C. perfringens* neuraminidase per 1.9 cm^2 of well surface area. Where indicated, bacteria were incubated with purified asialo-GM1 for 30 min on ice. Values are the mean of at three independent experiments ±SD.

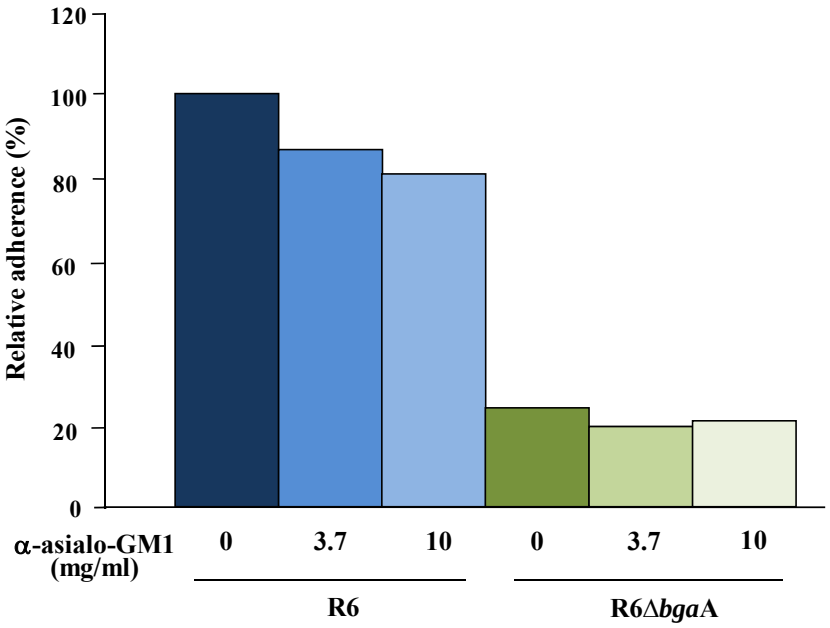

Figure 3. Anti-asialo-GM1 does not significantly reduce adherence of R6 to epithelial cells. Adherence of strains (O.D. 600 nm = 0.6) to D562 cells over 60 min is expressed as a percentage of parental strain adherence. All wells were pretreated for 30 min with 0.01 U of purified *C. perfringens* neuraminidase per 1.9 cm^2 of well surface area. Where indicated, wells were pretreated for 60 min at 37ºC with anti-asialo-GM1 polyclonal rabbit antibody. Values are the mean of at three independent experiments ±SD.

Differences in the contribution of BgaA to adherence is most likely not due to allelic variation.

To investigate possible allelic variation among isolates used to demonstrate clinical relevance, the *bgaA* allele of NCH18, NCH34, and NCH39 was sequenced and compared with the known sequence for the unencapsulated lab strain, R6. Amino acid variation between strains range from 0.9-1.9 %. These differences exist between strains that were reduced in adherence with the addition of rBgaA (R6 and NCH18) and those that were not (NCH34 and NCH39). The differences are located near the C-terminal region between amino acid 1760 and 2192. Most notably, R6 and NCH18 lack five amino acids found within NCH34 and NCH39 (2050-2055). The presence of conserved amino acid differences supports the hypothesis that the contribution of BgaA to adherence could be determined by allelic variation among strains.

The *bgaA* allele was exchanged between R6 and NCH39 by transformation with chromosomal DNA as a means to test this hypothesis. The NCH *bgaA* allele was transformed into the R6 background thereby creating R6*bgaA*NCH39 and the R6 *bgaA* allele was transformed into the NCH39 background thereby creating NCH39*bgaA*R6. A change in adherence phenotype for these strains would support the hypothesis. If R6*bgaA*NCH39 is not reduced in adherence to epithelial cells after the addition of rBgaA like the parental strain R6 and NCH39*bgaA*R6 is reduced in adherence to epithelial cells after the addition of rBgaA unlike the parental strain NCH39 then this would support the hypothesis that these allelic differences account for differences in the ability of BgaA to act as an adhesin. Similarly, a reduction in adherence of R6*bgaA*NCH39 without the addition of rBgaA would also support this hypothesis, as the allele would be unable to mediate adherence. However, testing these strains in adherence to epithelial

cells after the addition of rBgaA resulted in data that does not support this hypothesis. Neither strain showed an exchange in phenotype as adherence of the mutants was not significantly different and the ability to reduce adherence with rBgaA was the same as parental strains (Figure 4). This data suggest that the amino acid differences that exist between the *bgaA* alleles of strains do not account for differences in the ability of BgaA to act as an adhesin. Other reasons must therefore account for differences in the contribution of BgaA to adherence of clinical isolates to epithelial cells, such as protein expression level differences.

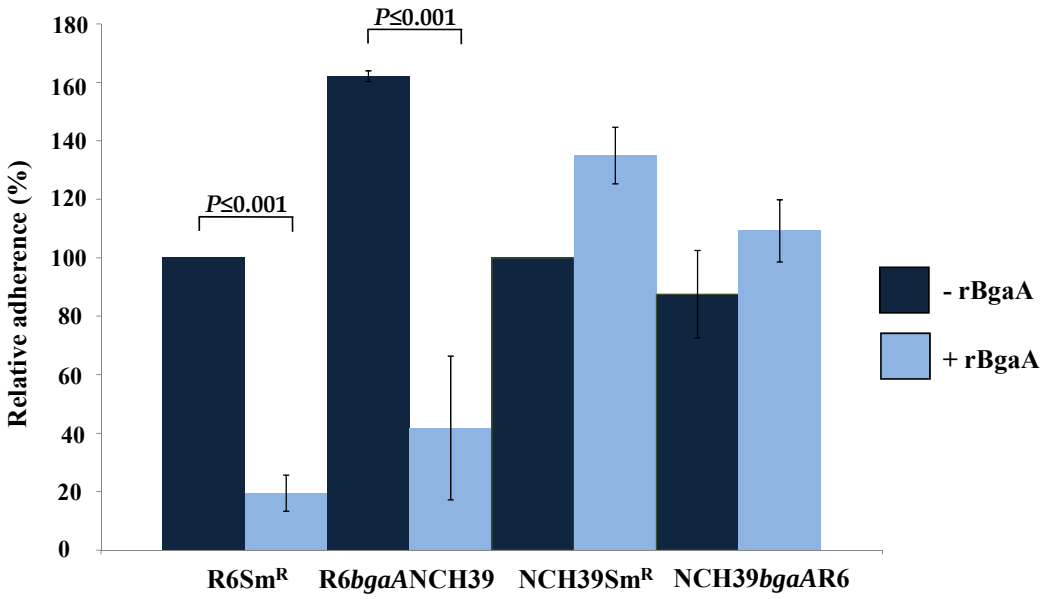

Figure 4. Demonstration that the exchange *bgaA* alleles does not change the adherence phenotype of R6 or NCH39. Adherence of strains (O.D. 600 nm = 0.6) to D562 cells over 60 min is expressed as a percentage of parental strain adherence. All wells were pretreated for 30 min with 0.01 U of purified *C. perfringens* neuraminidase per 1.9 cm^2 of well surface area. Where indicated, 0.433 pm of rBgaA was added to the inoculum. Values are the mean of at least three independent experiments ±SD. Statistical significance was determined using Student's *t*-test.

Differences in BgaA expression may affect the contribution of BgaA to adherence.

To investigate whether possible protein expression level differences affect the contribution of BgaA to adherence, β-galactosidase assays were performed using the clinical isolates tested in adherence. If expression levels do affect the BgaA-mediated mechanism of adherence, R6 and

NCH18 which were reduced in adherence after the addition of rBgaA would have higher expression (helping mediate adherence) and would therefore likely have higher enzymatic activity than NCH34 and NCH39 which were not reduced in adherence after the addition of rBgaA. Data support this hypothesis as R6 and NCH18 were found to have statistically significant higher β-galactosidase activity than NCH34 and NCH39 (Figure 5). Activity assays are merely an indication that there may be differences in protein expression levels and further investigation is required to fully support this hypothesis. R6*bgaA*NCH39 and NCH39*bgaA*R6 were also tested for β-galactosidase activity levels as a means to determine if the changes introduced in the *bgaA* alleles of the strains affected the activity of BgaA. Both strains were found to have similar activity to the respective parental strains, indicating that the changes introduced did not dramatically affect the enzymatic function of BgaA (Figure 6).

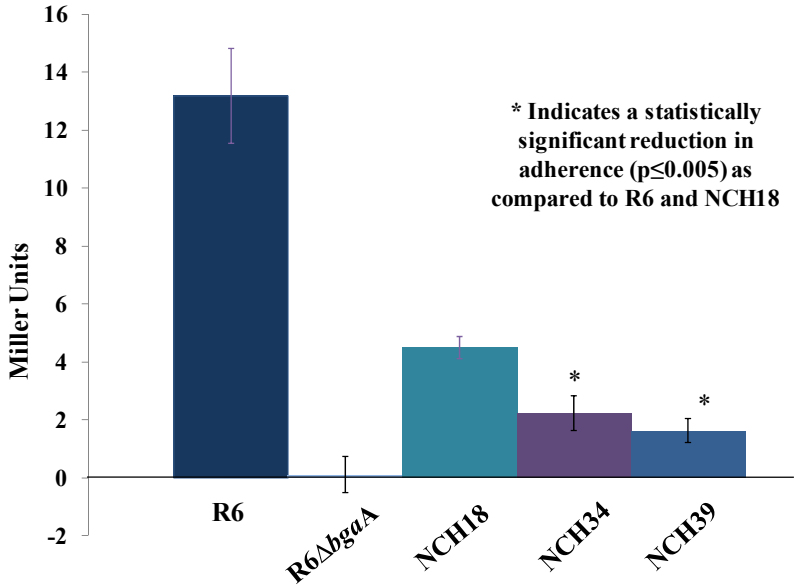

Figure 5. R6 and NCH18 have significantly higher β-galactosidase activity than NCH34 and NCH39. Strains were grown in THY broth (O.D. 600 nm = 0.6). Beta-galactosidase activity was determined using the colorimetric substrate O-Nitrophenyl beta-D-galactopyranoside. A media alone control was subtracted from all data. Values are the mean of three independent experiments ±SD. Statistical significance was determined using Student's *t*-test.

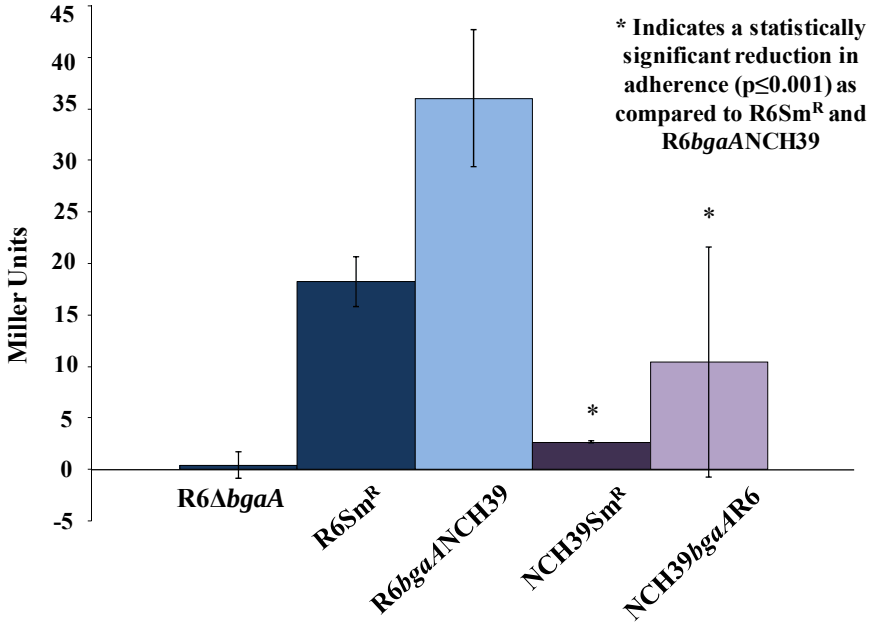

Figure 6. R6Sm^R and R6*bgaA*NCH39 have significantly higher β-galactosidase activity than NCH39Sm^R and NCH39*bgaA*R6. Strains were grown in THY broth (O.D. 600 nm = 0.6). Beta-galactosidase activity was determined using the colorimetric substrate O-Nitrophenyl beta-D-galactopyranoside. A media alone control was subtracted from all data. Values are the mean of three independent experiments ±SD.

DISCUSSION

The findings of this study help to further characterize this novel BgaA-mediated mechanism of adherence. Previous studies have determined that the receptor for BgaA is most likely a glycoconjugate as NanA acts to cleave sialic acid thereby revealing a receptor to which BgaA binds. Protease treatment resulted in an increase in adherence of R6 to epithelial cells determining that the receptor for BgaA is not a glycoprotein that can be cleaved by protease. The protease could be acting to further reveal a receptor for *S. pneumoniae* which could account for the observed increase in adherence. Although not all glycoproteins can be discounted, this data indicates that the epithelial cell receptor for BgaA is likely a glycosphingolipid. Asialo-GM1 has previously been proposed a receptor for *S. pneumoniae* (20). Our studies, however, show that this glycosphingolipid is most likely not the epithelial cell receptor for BgaA as R6 was not reduced in adherence after the addition of either purified GM1 or anti-asialo-GM1 which would serve to block the possible receptor. Together this data suggest that the receptor for BgaA is most likely a glycosphingolipid, but not asialo-GM1. Further investigation may help elucidate a possible receptor for BgaA.

As previous studies have demonstrated that BgaA contributes to the adherence of only some clinical isolates, investigating reasons for these differences may help to give insight into the BgaA-epithelial cell interaction including the identification of the possible binding site of BgaA and the discovery of a possible receptor. Allelic variation of *bgaA* was found between strains that were reduced in adherence after the addition rBgaA and those that were not. These differences are located at the C-terminal, a region of unknown function that may contain the binding site. The exchange of *bgaA* alleles between R6 and NCH39 was found to have no affect

on the contribution of BgaA to adherence as R6*bgaA*NCH39 and NCH39*bgaA*R6 had a similar adherence phenotype to the respective parental strains. Because chromosomal DNA was used for transformation, some DNA outside of *bgaA* may have also been exchanged. More time would allow for further investigation into where this exchange occurred; however, no changes made to the genome of either strain changed adherence phenotype and therefore differences in *bgaA* are most likely not responsible for the difference in contribution of BgaA to adherence.

Despite these findings, possible differences in the level of protein expression could account for the difference in the ability of BgaA to act as an adhesin. In investigation, R6 and NCH18 were found to have higher β-galactosidase activity than NCH34 and NCH39 which could indicate that BgaA expression level differences exist between clinical isolates and that a higher expression level may help to mediate adherence. R6*bgaA*NCH39 and NCH39*bgaA*R6 also showed no change in β-galactosidase indicating that the introduced amino acid changes did not significantly change the enzymatic activity of BgaA for each strain and that the difference in contribution of BgaA to adherence may be due to BgaA expression level differences. This hypothesis requires further investigation using Western blotting or real time-reverse transcriptase PCR as activity levels only suggest possible differences in expression level.

Capsule expression could also play a role in the contribution of BgaA to adherence, as a reduced amount of capsule correlates with an increase in colonization (34) which may be due to increased exposure of bacterial adhesins. Ultimatley, the distribution of other adherence mechanisms could affect differences in the contribution of BgaA to adherence among clinical isolates. Given the importance of adherence to colonization, multiple redundant mechanisms of adherence are

likely, all of which may contribute differently to adherence as is seen among the tested clinical isolates. The role of BgaA in adherence is novel and relevant in recent clinical isolates, however, more investigation is required for further characterization of this mechanism and understanding of how knowledge of this mechanism can aid in the development of preventative measure for pneumococcal adherence and colonization.

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